Production, Characterization and Immobilization of thermo-alkali-stable xylanase from *Geobacillus thermodenitrificans* X1 and its application in paper pulp bio-bleaching

SYNOPSIS

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Introduction

Xylanases [EC 3.2.1.8] are one of the ligocellulases that specifically act on β-1,4-xylosidic linkage between the two adjacent xylose residues in complex xylan structure [1]. Xylan is the second most abundant lignocellulosic component after cellulose, thus xylanases an effectively cause hydrolysis of diverse lignocellulosic residues such as wood and forest residues, agricultural residues and landfill wastes. The diverse sources of xylanases constitute bacteria, fungi, arthropods, protozoa, gastropods and actinomycetes. Among all, bacteria and fungi are the leading sources that are exploited on commercial scale for xylanase production [2]. Especially, thermophilic bacterial and fungal sources are preferred over the mesophilic. Hot springs are generally considered to be hub of thermophilic microbial diversity [3].

Xylanases may be intracellular or extracellular in nature [4]. Majority of bacterial xylanases are extracellular in nature which makes them commercially viable due to easy downstream processing. The mechanism of action of xylanases is a hydrolytic reaction, involving an acid-base catalyst and a nucleophile [1]. For production of xylanases, submerged and solid state fermentation is practiced under optimized physico-chemical conditions. For maximum xylanase production under specific conditions, the influencing parameters need to be optimized. Parameters such as temperature, pH, agitation speed rate, nitrogen sources, nitrogen concentration, substrate concentration etc. have influential impact on production of xylanase. Various optimization methods such as one-factor-at-a-time (OFAT) and Response Surface Methodoloy (RSM) are opted for optimizing the influencing parameters for xylanase production.

For industrial use, the enzyme must have high reusability, ease of recovery and high operational stability. Immobilization of enzymes is a promising alternative to improvise the efficacy of enzymes and enhance their applicability at commercial scale [5,6]. Various disadvantages have been associated with recent immobilization techniques such as adsorption, entrapment, encapsulation, covalent or non-covalent (affinity interactions) binding to solid-carrier. Immobilization through adsorption of enzymes confronts the problem of leakage from carrier material whereas binding to carrier material through affinity interactions involves recombinant tagged enzymes which make the method unsuitable for industrial use [7]. Similarly, covalent interactions with the carrier require expensive modifications of the carrier surface. On the contrary, carrier free immobilization through the formation of cross-linked enzyme

aggregates (CLEAs) offers various advantages. CLEAs can be easily separated by centrifugation or filtration of the reaction mixture, have low-cost preparation, recoverability/reusability and higher stability [8]. Also, the method is highly economical on a commercial scale due to easy scale-up and no requirement of the purified enzyme. Use of CLEAs allows feeding of the industrial reactors with a higher amount of enzyme as no space is compensated by the immobilization support.

Xylanases serve various industries such as food and feed industry, pharmaceutical industry, biofuel industry and paper industry. In food industry, xylanases aid in reducing the viscosity and increasing the clarity of juices and thus enhance their quality [9]. Addition of xylanases in poultry diet increases the nutritional value and xylooligosaccharides are used in many feed products [10]. In biofuel industry, xylanases help in lignocellulosic biomass conversion by hydrolyzing the hemicellulosic component into sugars, which could be further fermented into biofuel [11]. In paper industry, xylanases act on hemicelluloses component of paper pulp and thus breaks the bonds between hemicellulose and lignin. This makes the lignin molecules easy accessible to other bleaching agents and get the increased brightness of paper [12]. Moreover, it has been reported that use of xylanases for biobleaching leads to reduction in consumption of chemical bleaching agents and also enhance the quality of paper. In this context, the present study was also focused on application of xylanase for paper pulp biobleaching.

So, far bacterial xylanases have been in more demand in comparison to fungal xylanases. The xylanases from fungi, usually have pH optima in neutral or acidic range and are active at low temperatures. Also, cost effective strategies have to be inculcated to reduce the production cost of enzymes to make them commercially viable. To be applicable in paper industry, xylanases need to be thermo-alkali-stable and cellulase free. With respect to these research gaps, the present study was conducted with the following objectives:

Objectives

1. Screening of potent thermophilic xylanolytic bacteria from Tattapani hot spring of Himachal Pradesh
2. Production, parametric optimization and characterization of xylanase from *Geobacillus thermodenitrificans* X1
3. Synthesis and characterization of cross-linked enzyme aggregates (CLEAs) of xylanase
4. Application of xylanase for biobleaching of pulp in paper industry

Objective 1: Screening of potent thermophilic xylanolytic bacteria from Tattapani hot spring of Himachal Pradesh

With an aim to get potent thermo-alkali-stable xylanase, we targeted at finding thermophilic bacterial sources. Since, bacterial xylanases have been reported to show higher thermostability and alkaline stability in comparison to fungal xylanases, so our study was focused on isolation of only bacterial thermo-xylanolytic strains. Geothermal areas such as hot springs are bestowed with diverse thermophilic ecology; thus, sample collection for isolation of thermophilic bacteria was done from hot spring. Various thermophilic bacterial isolates were screened for xylanase activity and the isolate with maximum xylanase activity was used for further study.

Materials and methods

Media enrichment, isolation and screening of xylanolytic bacteria

Soil sample was collected in sterile containers, from Tattapani hot spring (District Mandi, Himachal Pradesh, India) as a potential source for thermophilic xylanolytic bacteria. The hot spring has been submerged in the past years and does not exist at present. The soil sample was stored at 4°C in laboratory. One g of soil sample was added to 100 mL of minimal medium [13] at pH 7, containing 0.1% (w/v) beechwood xylan, followed by incubation at 60°C. After 72 h, 1 mL of liquid was transferred to the fresh medium and was followed by 3 to 4 such sequential transfers. Aliquot from enriched medium was serially diluted and spread on xylan (0.1% w/v) agar plates, incubated overnight incubation at 60°C and then screened for xylanase activity.

Primary Screening by Congo red assay:

Thermophilic bacterial isolates were grown for 24 h in nutrient broth at 60°C, 150 rpm. After, 24 h, culture supernatant was collected and put in the wells punctured on xylan agar plates. The xylan plates were incubated at 60°C for 48 h, followed by staining with congo red dye (0.1%) [14] for 15 min and then destaining with 1M NaCl to observe the zone of hydrolysis.

Secondary Screening by DNS method:

Bacterial isolates were grown in nutrient broth at 60°C for 24 h at 150 rpm. Inoculum of each culture was inoculated in minimal medium with 0.1 % bechwood xylan, separately. Xylanase production was carried out at 60°C for 48 h at 150 rpm. The production medium was centrifuged

at 10,000 rpm for 10 min and supernatant collected was assayed for xylanase activity by using 3,5-dinitrosalicylic acid DNS reagent [15].

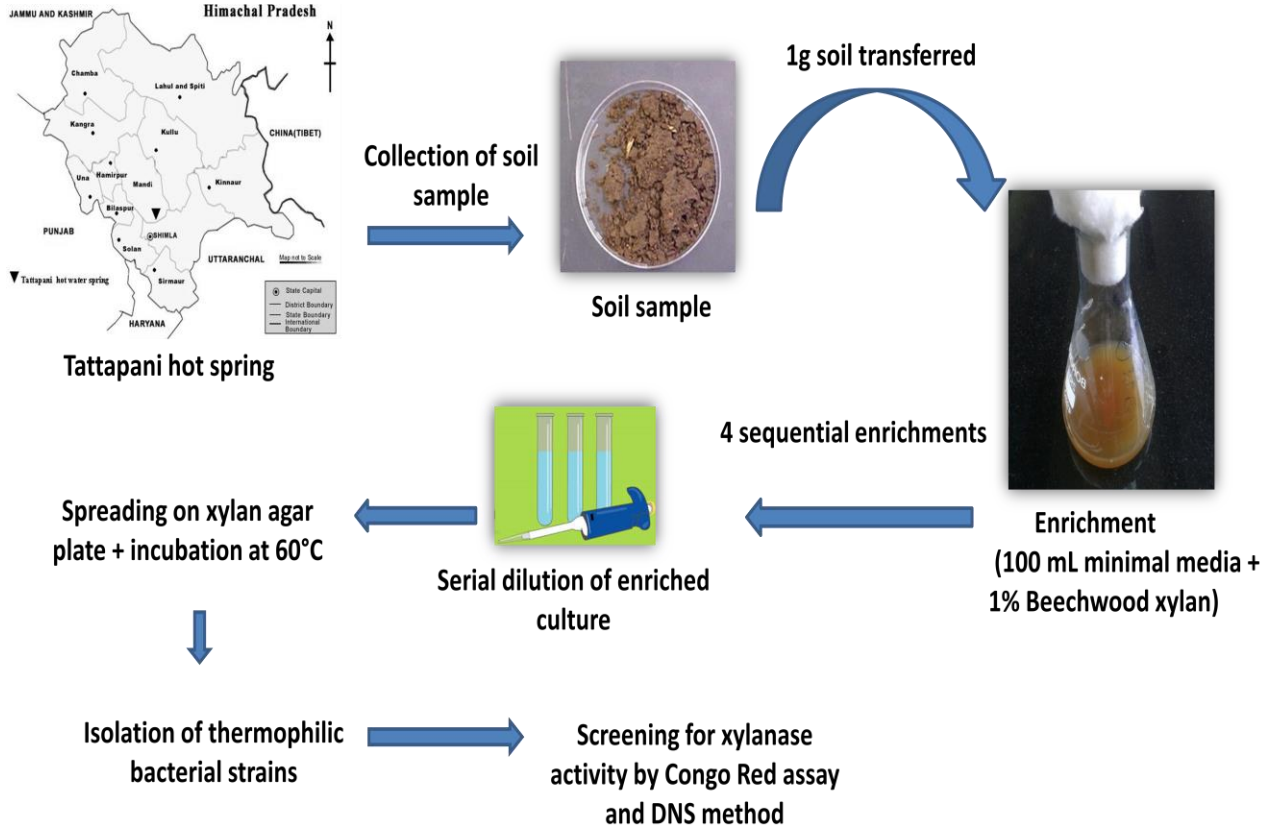


Figure1 Schematic representation of sample collection, isolation of thermophilic xylanolytic bacteria by enrichment culture technique, followed by screening of potent xylan degrading bacteria.

Xylanase Activity Assay

The xylanase activity assay was performed by incubating 0.5mL of 0.5%(w/v) xylan in 100 mM sodium phosphate buffer, pH 7 with 0.5 mL of appropriately diluted crude enzyme at 70°C for 5 min. After incubation, 1.5 mL of DNSA reagent was added to stop the reaction, followed by boiling for 10 min and then cooled on ice to stabilize the color. Optical absorbance was read at 540 nm for measuring the amount of xylose. "One unit (U) of xylanase activity was defined as the amount of enzyme required to release 1 µmol of xylose-equivalent reducing groups per min under assay conditions".

Identification of Bacterial isolate

Most potent xylanolytic bacterial isolate was subjected to morphological characterization by studying colony characteristics, gram staining and KOH string test. For molecular identification, 16S rRNA gene sequence was amplified using universal primers 27F(5'-AGAGTTTGATCMTGGCTCAG, where M is A or C) and 1492R (5'-ACCTTGTTACGACTT) .The sequenced PCR product was compared with similar bacterial sequences using NCBI BLAST, followed by phylogenetic analysis using MEGA 4 [16].

Results and discussion

Isolation, Screening, and Identification of xylanolytic isolate

Four xylanolytic bacterial isolates (X1, X2, X3 and X4) were obtained from hot spring soil sample. After primary srceening by congo red assay, X1 isolate showed maximum zone of hydrolysis (30 mm) as shown in Fig.2a. In secondary screening, X1 isolate showed maximum xylanase activity of 2.3 U/mL. Colony morphology of X1 isolate showed irregular shape with an entire margin, flat and viscous surface, rough and off-white appearance, gram-positive and KOH positive. Through phylogenetic analysis (Fig.2b), X1 isolate was identified as *Geobacillus thermodenitrificans* accession no. KT 899095.

Figure 2 a) Congo red assay of X1 isolate **b)** A neighbor joining phylogenetic tree based on the 16S rRNA gene sequence of *Geobacillus thermodenitrificans* (KT899095)

Objective 2: Production, parametric optimization and characterization of xylanase from *Geobacillus thermodenitrificans* X1

This objective was aimed at cost-effective production of xylanase under optimum conditions. Various influencing parameters were optimized to attain maximum xylanase production from *Geobacillus thermodenitrificans* X1, under submerged fermentation conditions. Cheap lignocellulosic substrates such as wheat straw, rice straw, corn cob and wheat bran were valorized for cost-effective xylanase production. Two optimization methods, namely one-factor-at-a-time (OFAT) and response surface methodology were practiced. To know about the best conditions under which xylanase could show its maximum activity, characterization in terms of temperature and pH optima, thermostability, pH stability and effect of metal ions was studied. Thermostability and pH stability were the important characteristics for analyzing the applicability of xylanase in paper pulp biobleaching.

Materials and methods

Parametric optimization for Xylanase Production by One-Factor-At-a-Time (OFAT) and Response Surface Methodology (RSM)

The OFAT optimization process was initiated at 60°C, with 1% (v/v) inoculum size (~$3.0*10^7$ CFU/mL) and 150 rpm, for 48 h. The fermentation medium was centrifuged at 10,000 rpm for 5min and supernatant collected for xylanase activity assay. The optimized parameter was then utilized in the succeeding optimization step. In all the optimization experiments, a set of control experiment was run with conditions of the preceding optimization step. The parameters studied were inoculum size (0.5% to 2%, v/v), incubation time (24 h to 120 h), temperature (40°C to 60°C), pH (4 to 10), beechwood xylan concentration (0.5% to 3%), a single nitrogen sourceas a replacement for yeast extract(peptone, beef extract and urea at a concentration of 0.005 g/L), variable concentration of nitrogen source (0.005 g/L to 5 g/L) and agitation speed rate (50 to 200 rpm). In addition, cheap substrates such as wheat straw, rice straw, corn cob and wheat bran at 1% (w/v) concentration were exploited for xylanase production. The significant parameters selected from OFAT were subjected to statistical optimization through Response surface methodology. For RSM, central composite design (CCD) was created using software Design

expert 10. CCD design generated 20 experimental runs comprising of 6 trials for axial points, 8 trials for factorial points and 6 replicate trials at central points. For all the respective trials of the independent variables xylanase activity (U/mL) was the response value, are shown in Table 1. The response value produced from each CCD trial was the average of duplicates. Statistical parameters were analyzed using Analysis of Variance (ANOVA). For validation of model, the optimum conditions predicted by the model were employed for production of xylanase.

Characterization of xylanase

The optimum pH for the xylanase activity was determined at different pH ranging from 3-10. The assay buffers used were sodium citrate (100 mM, pH 3–5), sodium phosphate (100 mM, pH 6–7), Tris-HCl (100 mM, pH 8-9), and glycine–NaOH (100 mM, pH10). For determining the optimum temperature, activity assay was performed at different temperatures ranging from 30°C to 90°C at an optimum pH. Thermostability of the crude enzyme was estimated by pre-incubating the enzyme solution in 100 mM Tris-HCl (pH 8) at 50°C, 60°C and 70°C for 4 h. Residual xylanase activity was then determined at the standard conditions. A control was run in parallel without any pre-incubation. For studying the pH stability, the crude enzyme was pre-incubated in different pH buffers (3-10) at 70°C for 4 h whereas crude enzyme without any pre-incubation was referred as a control. After that, estimation of residual activity was done by the standard DNS method. Effect of various metal ions was studied by preincubating the crude xylanase with a metal ion (10mM) at 70°C for 30 min; no metal ion was added in control. Residual xylanase activity was then determined at the standard assay conditions.

Hydrolysis of beechwood xylan was carried by incubating 20 U xylanase/g xylan [17] in Tris-HCl buffer (100mM, pH 8) at 60°C, 150 rpm for a duration of 48 h. No xylanase was added in the control for the experiment. After 48 h, various hydrolysis products produced were analyzed through high performance liquid chromatography (HPLC) using refractive index (RI) detector and Aminex HPX-87P column (300 X 7.8 mm; column). 20 µL of sample was injected and the analysis was done at column temperature of 80°C with a flow rate of 0.6 mL/min, using Milli-Q water as the mobile phase.

Gene length and molecular weight of xylanase from *Geobacillus thermodenitrificans* X1 (GTX1) was also determined. Reverse and forward primers were designed using Primer 3 tool

for amplification of xylanase gene from GTX1. For molecular weight determination, zymogram of GTX1 xylanase was performed. For, zymogram crude xylanase was concentrated using Amicon Ultra-15-Millipore (10 kDa cut off). Concentrated xylanase was mixed with SDS sample buffer and heated at 95°C for 5 min, prior to loading on gel. Sample was loaded on 12% SDS PAGE gel, followed by cutting the gel such that the lane containing protein ladder is separated from the gel containing crude xylanase. The ladder containing gel was stained with coomassie dye. On the other hand, for zymogram the gel with crude xylanase was given three successive washings of 20 min each with 20% isopropanol. After that gel was immersed for 1 h at 60°C, in sodium phosphate buffer (50 mM, pH 7) containing 1% beechwood xylan. Incubation was followed by staining with 0.1% congo red dye for 15 min and destaining with 1M NaCl.

RESULTS AND DISCUSSION

Parametric optimization for Xylanase Production from *Geobacillus thermodenitrificans* X1

Effect of Inoculum Size and Incubation Time

The inoculum size of 1.5% (v/v) and incubation time of 96 h was found optimum for xylanase activity (Fig.3a and 3b). Maximal production of xylanase during stationary phase might be due to outflux of traces of intracellular xylanase into the production medium after autolysis of the aged cells [17,18]. Whereas, increasing the incubation time further (beyond 96 h), did not increase the enzyme production. This could be due to exhaustion of nutrients towards later stages of fermentation. Xylanases from *Geobacillus* sp. Strain WSUCF1 [17] and *Bacillus* SSP-34 [2] also showed maximum production after 96 h.

Effect of Temperature and Initial pH

The optimum temperature for xylanase production was found to be 50°C, resulting in the production of 12.15 U/mL of xylanase activity (Fig.3c). The optimum pH range for xylanase production was observed between pH 7-8. The pH profile of xylanase production by *Geobacillus sp.* showed drift towards alkaline region as shown in Fig. 2d. The pH profile clearly depicted that enzyme production by a particular bacterium is affected by variations in pH of the medium. Any variations in the cytoplasmic pH can cause disruption of the plasma membrane and inhibition of metabolic activities. The ionization state of the nutrient molecules varies with pH variations,

thereby decreasing their accessibility to microbial cells [3]. Similar to our findings, a pH of 8 was reported optimum for xylanase synthesis for *Bacillus megaterium* [19] and *Bacillus mojavensis* AG137 [18].

Effect of Substrate Concentration

Among different tested concentrations of xylan as a carbon source for xylanase production, 1 to 1.5% (w/v) xylan was found to be optimum for maximum enzyme production (Fig.3e). With increasing xylan concentration (1%, w/v), there was a significant increase in the enzyme production, which justifies the inductive nature of xylanase produced. However, at higher substrate concentration, an inducer (xylan) is capable of enhancing xylanase productionup to a certain limit beyond which the amount of enzyme formed may remain constant or even decline due to reasons like product or feedback inhibition [19].

Effect of Nitrogen Sources and Concentration

Among various nitrogen sources, both peptone and yeast extract were found to be an efficient nitrogen sources for xylanase production, producing xylanase activity of 14.3U/mL and 13.75 U/mL, respectively(Fig.2f). On varying the concentration of peptone, the range of 0.005-0.05 g/L was found optimum for xylanase activity (Fig.3g). The choice of nitrogen source for maximum xylanase production varies among different bacteria [20].

Effect of Agitation Speed Rate

Maximum xylanase production was observed at 200 rpm with the unit activity of 18.32 U/mL (Fig.3h), due to better aeration and uniform distribution of nutrients at higher agitation rate [21]. The lower enzyme production at low agitation rate is attributed to the limited dissolved oxygen available for cell growth and cell aggregation due to improper mixing [18]

Effect of Different Carbon Sources

Among the different carbon sources tested, wheat straw and beechwood xylan proved to be the best carbon source showing xylanase activity of 18.5 U/mL and 18.2 U/mL (Fig.3i). Since wheat straw is the second most abundant lignocellulosic residue in the world, it is a promising option as a raw material on a commercial scale [22,23].

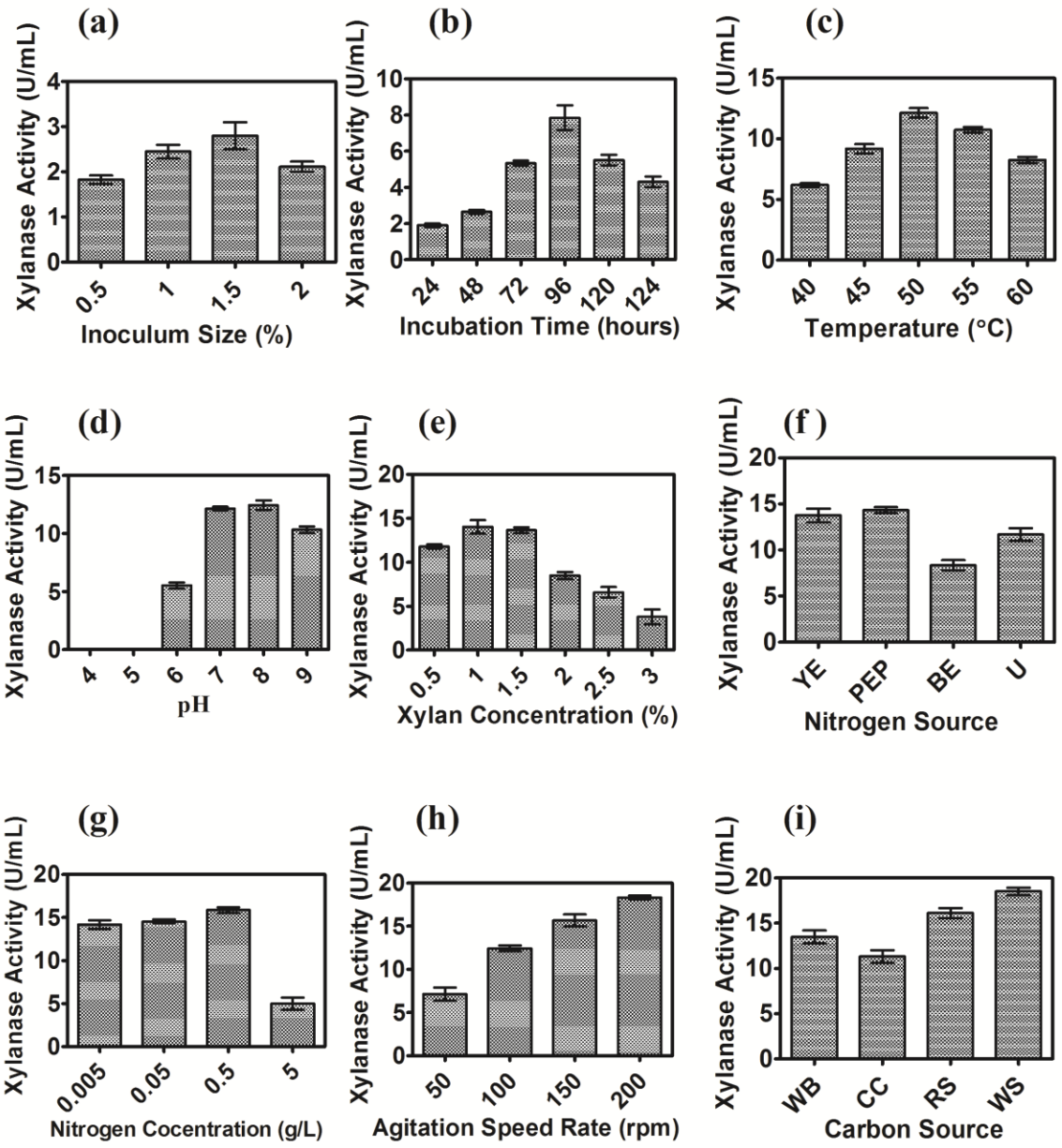


Fig. 3 a) Effect of inoculum size (0.5 to 2%) on xylanase production at 60˚C, 150 rpm for 48 h. Minimal media with pH 7, 0.005 g/L yeast extract and 0.5% beechwood xylan was used. All values are mean of two replicates ± SE. **b)** Effect of incubation time (24 to 124 h) on xylanase production at 60˚C and 150 rpm using 1.5% inoculum. Minimal media with pH 7, 0.005 g/L yeast extract and 0.5% beechwood xylan was used. **c)** Effect of temperature (40 to 60˚C) on xylanase production at 150 rpm for 48 h using1.5% inoculum. Minimal media with pH 7, 0.005 g/L yeast extract and 0.5% beechwood xylan was used. **d)** Effect of pH (4 to 9) on xylanase production at 60˚C, 150 rpm for 48 h using 1.5% inoculum. Minimal media with pH 7, 0.005 g/L yeast extract, 0.5% beechwood xylan was used. **e)** Effect of xylan concentration (0.5 to 3%) on xylanase production at 60˚C, 150 rpm for 48 h using 1.5%inoculum. Minimal media with pH 8, 0.005 g/L yeast extract, 0.5% beechwood xylan was used. **f)** Effect of nitrogen sources other than yeast extract (YE), such as peptone (PEP), beef extract (BE) and urea (U) at a concentration of 0.005 g/L on xylanase production at 60˚C, 150 rpm for 48 h using 1.5% inoculum. Minimal media with pH 8, 0.5% beechwood xylan was

used. **g)** Effect of nitrogen concentration (peptone at concentrations 0.005 to 5 g/L)on xylanase production at 60°C, 150 rpm for 48 h using 1.5% inoculum. Minimal media with pH 8, 0.5% beechwood xylan was used. All values are mean of two replicates ± SE.

Optimization of xylanase production by RSM

The three input variables, i.e., temperature, incubation time and agitation speed rate were the selected significant parameters. The response (xylanase activity) with respect to 20 different trials is listed in Table 1. Run 11 showed the highest xylanase activity (23.5 U/mL) while run 16 showed minimum with, no xylanase production.

Table 1 Factors and levels in the response surface central composite design arrangement along with the experimental and predicted results

				Response (Xylanase Activity)	
Run	A	B	C	Experimental (U/mL)	Predicted (U/mL)
1	55	80	150	22.9	22.5
2	55	80	150	21.7	22.5
3	70	40	200	2.5	4.8
4	55	80	150	22	22.5
5	55	12.8	150	0.7	0
6	70	40	100	1.02	1.6
7	55	80	65.9	16.5	14.3
8	40	40	200	12.2	11.8
9	70	120	200	0.9	1.8
10	55	80	150	22.5	22.5
11	55	80	150	23.5	22.5
12	40	40	100	0.9	2.7
13	55	80	234.1	21.8	20.2
14	29.8	80	150	12	11.1
15	40	120	100	13	13.4
16	80.2	80	150	0	0
17	40	120	200	15.2	17.3
18	70	120	100	0.7	3.8
19	55	80	150	21.8	22.5
20	55	147.3	150	8.5	5.9

A: Temperature (°C), B: Incubation time (hour) and C: Agitationspeed (rpm)

By applying multiple regression analysis, a second-order polynomial equation was generated. The final coded equation was as follows:

Xylanase activity (U/mL) = +22.48 - 4.72A + 1.33B + 1.19C – 1.13AB - 0.49AC – 0.30BC - $6.30A^2 – 6.79B^2 – 1.65C^2$

Where A is temperature (degree Celsius), B is incubation time (hour) and C is agitation speed rate (rpm). Table 2 shows the data obtained from ANOVA for Response Surface Quadratic model. The model F-value was 34.81 and value of ‘‘Prob>F’’ was less than 0.05; both values imply that the model is significant.

The ANOVA data showed that A, B, C, AB, A^2, B^2 and C^2 were significant model terms. This showed that temperature and incubation time have crucial impact on the xylanase production as they have significant influence on the production at the linear and quadratic level as well as at the interactive level. A good correlation was found between the observed values and the predicted values as the value for “determination coefficient” R^2 is 0.9691; which is close to unity. It shows that the model could explain 97% of the variability.

Table 2 ANOVA for response surface quadratic model (xylanase activity)

Source	Sum of Squares	df	Mean Square	F Value	*p*-value	Prob> F
Model	1596.9	9	177.4	34.8	<0.0001	Significant
A-Temperature	232.6	1	232.6	45.6	<0.0001	
B-Incubation time	50.6	1	50.6	9.93	0.0103	
C-Agitation	42.5	1	42.5	8.3	0.0162	
AB	36.2	1	36.2	7.1	0.0237	
AC	17.5	1	17.5	3.43	0.0939	
BC	13.47	1	13.47	2.64	0.1351	
A^2	610.1	1	610.1	119.7	<0.0001	
B^2	706.48	1	706.48	138.6	<0.0001	
C^2	49.72	1	49.72	9.75	0.0108	
Residual	50.97	10	5.1			
Pure Error	2.5	5	0.5			
Cor Total	1647.9	19				

A good correlation between the experimental and predicted values is marked by the value of R (correlation coefficient) closer to 1 [24]. The "Predicted R^2" of 0.7681 was in reasonable agreement with "Adjusted R^2" of 0.9412. The signal to noise ratio is measured by adequate precision value. A ratio greater than 4 is desirable and the ratio of our model was 15.876, indicating an adequate signal which means that this model can be used to navigate the design space. The value for "coefficient of variation" (CV) was low (18.79 %), which indicates a good precision and reliability of the experiments conducted [25].

Validation of the Model

The model predicted maximum xylanase production at 50˚C temperature, 87h incubation time and an agitation rate of 177 rpm. The predicted xylanase activity on these conditions was 23.9U/mL. On carrying out production at the optimum conditions, xylanase activity of 24 ± 2 U/mL was achieved.

Xylanase characterization

The optimum temperature for the xylanase activity was 70˚C (Fig.4a) as reported by Marcolongo et al. (2015) and Anand et al. (2013). The optimum pH was found to be 8 (Fig.4b), similar to that reported in case of other isolates of *Geobacillus thermodenitrificans* [26]. The enzyme showed appreciable stability at 60˚C and 50˚Cwith 90.8% and 99.9% residual activity, respectively after 4h (Fig.5a) and was stable in the broad range of pH; showing higher stability in alkaline range (Fig.5b). Among all the metal ions, Ca^{+2} ions showed maximum stimulation and Mn^{+2} ions showed maximum inhibition on xylanase activity (Fig.5c).

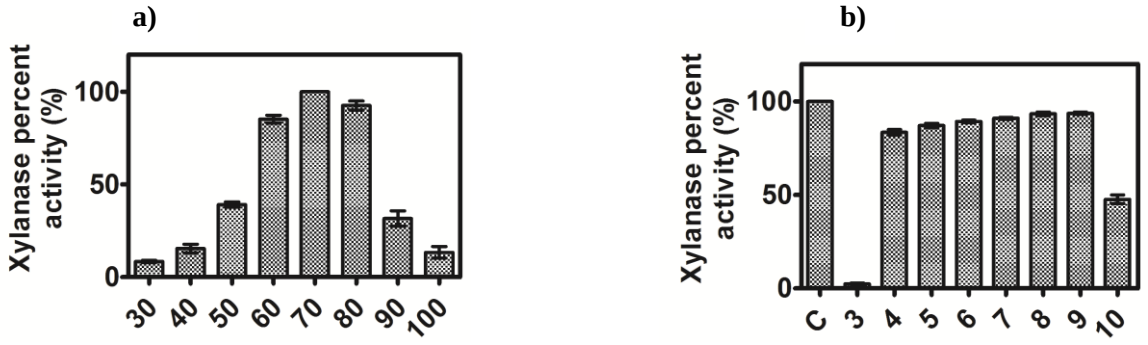


Figure 4 a) Effect of temperature (30˚C-90˚C) on xylanase activity at pH 7. Maximum activity (100%) was 24.8 U/mL **b)** effect of pH (pH 3-10): on xylanase activity at 70°C. Maximum activity (100%) was 25.3 U/mL**.** All values are mean of three replicates ± SE.

Xylanase from *Geobacillus thermodenitrificans* efficiently carried out hydrolysis of beechwood xylan at alkaline pH 8, producing xylose (0.22 ± 0.01 g/L), xylobiose (1.46 ± 0.02 g/L), xylotriose (1.64 ± 0.02 g/L) and xylotetrose (0.65 ± 0.01 g/L). The capability of producing XOs makes xylanase a reliable option for large-scale production of XOs; required in aquaculture and poultry industry. Moreover, enzymatic production of XOs is preferred over chemical methods. The sequencing of PCR product (amplified xylanase gene) revealed gene length of 1,224 bp which has been submitted to NCBI under the accession no. MG874777. Zymogram analysis of crude xylanase showed a light yellow band near 45 kDa, against the red background of congo red dye (Fig.5d). The molecular weight of GTX1 xylanase estimated through zymogram lies in the same range as reported for xylanase from other *Geobacillus thermodenitrificans* isolates [27,28].

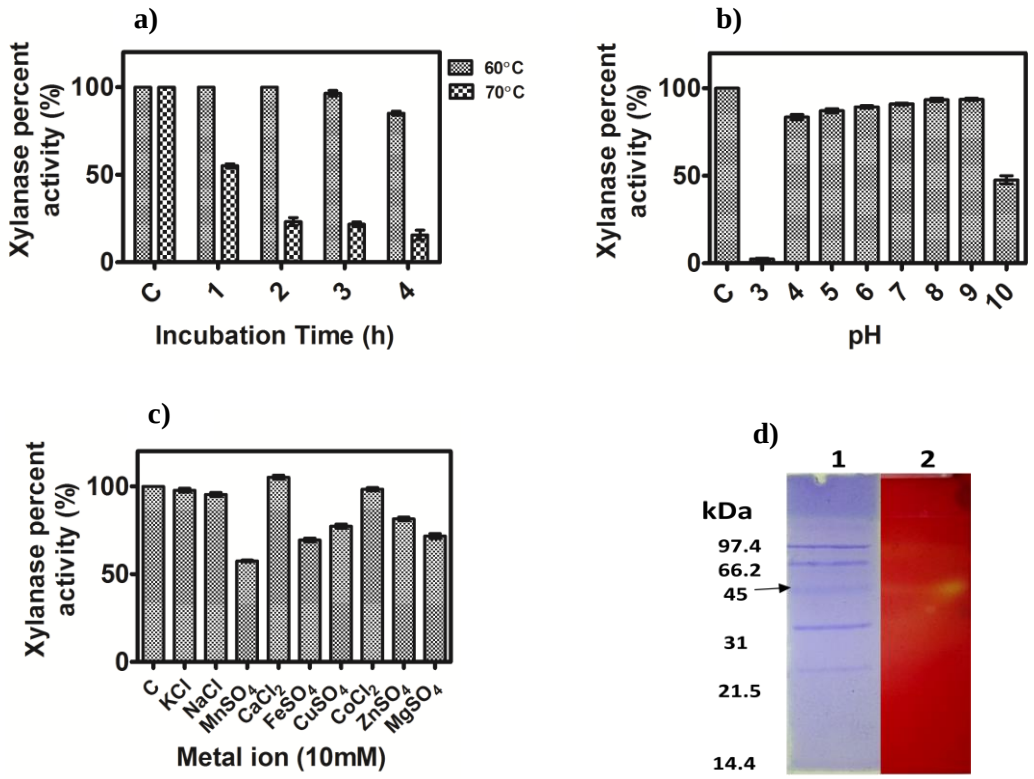


Figure 5 a) thermostability of xylanase at 50˚C, 60˚C and 70˚C at pH 8, with reference to the control (C) as 100% **b)** pH stability of xylanase at pH 3-10 and temperature 60˚C with reference to the control (C) as 100% **(c)** effect of metal ions (10mM) at pH 8 and 60˚C on xylanase activity; with reference to the control (C) as 100%. All values are mean of three replicates ± SE. **d)** Zymogram of xylanase; 1= ladder, 2= zymogram band of concentrated crude xylanase.

Objective 3: Synthesis and characterization of cross-linked enzyme aggregates (CLEAs) of xylanase

Immobilization plays significant role in enhancing the industrial viability of an enzyme by increasing its functionality and reusability. Thus, our objective was to immobilize crude xylanase to make it commercially viable. For this, carrier free immobilization technique, involving formation of xylanase cross-linked enzyme aggregates (CLEAs) was opted. Xylanase ClEAs were prepared and their characteristic features in terms of shape, size, temperature and pH optima, thermostability, pH stability, reusability and storage stability were studied. Also, the characteristics of xylanase CLEAs were compared with the free xylanase.

Materials and methods

Preparation of Xylanase CLEAs (Xy-CLEAs)

Ice-cooled acetone was added dropwise to 10 mL of crude xylanase (1.67 mg/mL) for the precipitation of xylanase molecules. The precipitation was carried out for 1 h under continuous stirring at 4°C, 15 rpm. After 2 h, GA (25% v/v) was added as cross-linker at a final concentration of 0.02% (v/v) for cross-linking xylanase aggregates. The cross-linking was carried out at 4°C, 150 rpm for 4 h. The Xy-CLEAs were recovered by centrifugation at 10,000 rpm for 5 min at 4°C followed by washing with sodium phosphate buffer (50 mM, pH 7) thrice [29]. CLEAs recovered were suspended in 50 mM sodium phosphate buffer (pH 7) and assayed for xylanase activity, followed by storage at 4°C.

Optimization of Xy-CLEA preparation

Various parameters such as, protein concentration (0.8-3.3 mg/mL), type of precipitant, protein: precipitant ratio (1:5-1:25), precipitation time (30-150 min), cross-linker concentration (0.005-0.05% v/v) and reaction time (2-10 h) were optimized by OFAT method.

Characterization of Xy-CLEAs

The Xy-CLEAs were lyophilized and subjected to SEM analysis to study morphology. The size of Xy-CLEAs was estimated through DLS analysis and alterations in the protein structure of Xy-

CLEAs were estimated through FTIR. The spectrum was studied within the wave number range of 4000 cm^{-1} to 500 cm^{-1}.

The optimum pH for the xylanase activity of free xylanase and CLEAs was determined at pH range from 3-10. The assay buffers used were sodium citrate (100 mM, pH 3-5), sodium phosphate (100 mM, pH 6-7), Tris-HCl (100 mM, pH 8-9), and glycine-NaOH (100 mM, pH 10). To determine the optimum temperature, xylanase activity was recorded by incubating reaction mixture in assay buffer of pH 7 (100 mM sodium phosphate) at 30, 40, 50, 60, 70, 80, 90 and 100°C.

Thermostability of the free xylanase and CLEAs was estimated by pre-incubating the free and immobilized xylanase in 100 mM Tris-HCl (pH 8) at 60°C and 70°C for 4 h. The reaction mixture without incubation was considered as control (100% percent activity). The kinetic parameters for thermal inactivation were obtained utilizing the experimental data in the equation reported as $\ln A/A_0 = K_d t$, where A_0 is the initial activity obtained without pre-incubation, A is the residual activity after heat treatment, K_d is thermal inactivation rate constant (h^{-1}) and t is the exposure time (h). The half-life of the xylanase ($t_{1/2}$, h) was determined from the relationship: $t_{1/2}=\ln 2/K_d$. To determine pH stability, the immobilized and free xylanase were incubated in different pH (3-10) at 60°C for 4 h whereas xylanase without any incubation was considered as a control (100% percent activity).

CLEAs were stored in 50 mM sodium phosphate buffer (pH 7) at 4°C for 8 weeks to estimate storage satbility. At regular intervals of 1 week, percent xylanase activity was calculated and 10% activity was considered as control. For estimating reusability, CLEAs were reused for consecutive six cycles of xylanase activity assay. Each cycle carried at 70°C for 5 min was followed by separation of CLEAs by centrifugation at 10,000 rpm for 5 min. The recovered CLEAs were washed twice with 50 mM sodium phosphate buffer (pH 7) and then reused in the consecutive cycle. The supernatant recovered in each cycle was assayed for xylanase activity by DNS method. The xylanase activity in the first cycle was considered as 100 %. The CLEAs recovered after the sixth cycle were washed and lyophilized for SEM analysis.

RESULTS AND DISCUSSION

Optimization of xylanase CLEA preparation

Optimization of protein concentration and type of precipitant

Protein concentration of 1.7 mg/mL was found optimum for CLEAs synthesis (Fig.6a). At higher protein concentrations decreased percent CLEA activity was observed. Enzyme crowding at high protein concentrations can reduce the enzyme mobility due to the interactions that occur when the chain of one enzyme will crash versus the surrounding protein molecules. Ultimately, the resulting CLEAs lose stability and have decreased activity [30]. The optimized concentration of protein (1.7 mg/mL) was then used to evaluate various organic solvents as precipitating agents. Relative percent of the precipitated protein was estimated with reference to the control (no addition of organic solvent). 100% xylanase activity was retained on using acetone as the precipitant in comparison to other organic solvents, as shown in Fig.6b. Easa et al. (2017) used 65% acetone for precipitation of amylase from *Zophobas morio* [29]. Whereas, Dalal et al. reported 100% precipitated activity for pectinase, xylanase and cellulase on using n-propanol as precipitant [31].

Protein: precipitant concentration

The protein: acetone ratio of 1:10 (v/v) was found to show the highest CLEA recovery (Fig.6c). Organic solvents facilitate the aggregation of proteins through electrostatic interactions. They displace water molecules around the protein surface and reduce the dielectric constant of the solution [32,33].

Precipitation time

Maximum CLEA activity was observed after precipitation time of 120 min (Fig.6d). The time required for precipitation varies among different proteins [34]. Literature reports depict that CLEAs of *Saccharomyces cerevisiae* invertase were precipitated within 30 min CLEAs of amylase from *Zophobas morio* took 16 h for precipitation [29,35].

Cross-linker concentration and reaction time

GA is the most commonly preferred cross-linker due to low cost and easy availability for the commercial purpose [35,36]. In the present study, GA concentration of 0.01% v/v (Fig.6e) and cross-linking time of 2 h (Fig.6f), were found optimum for maximum CLEA activity. Higher GA concentration resulted in decreased CLEA activity as reported earlier [37]. Similar trend was

observed on increasing cross-linking time. At higher cross-linking concentrations and time, there is higher interaction between the cross-linker and enzyme aggregates, which then decreases structural flexibility of enzyme molecules [34]. At lower cross-linker concentrations and short duration of cross-linking time; insufficient cross-linking and loss in CLEA activity occurs [36].

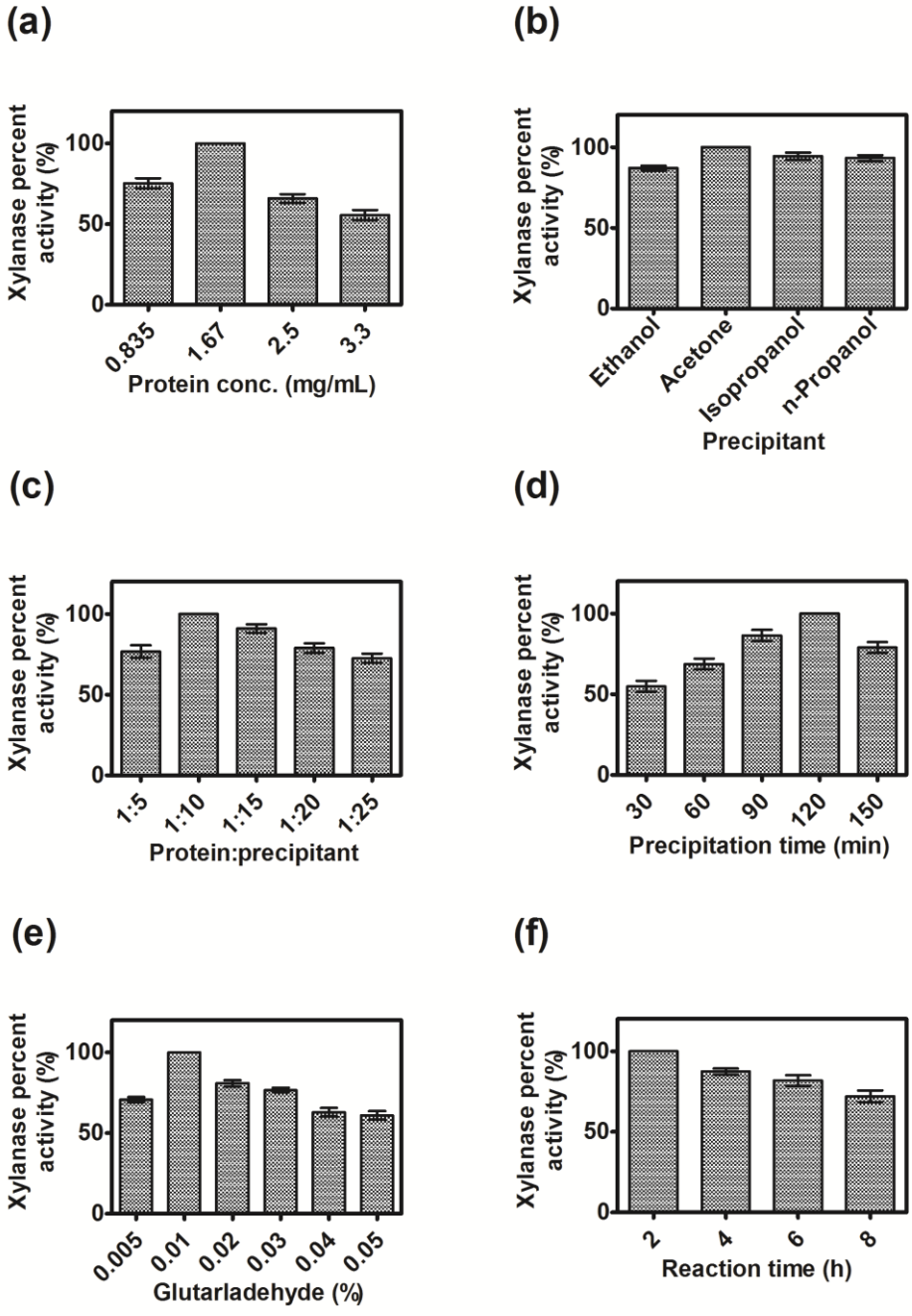
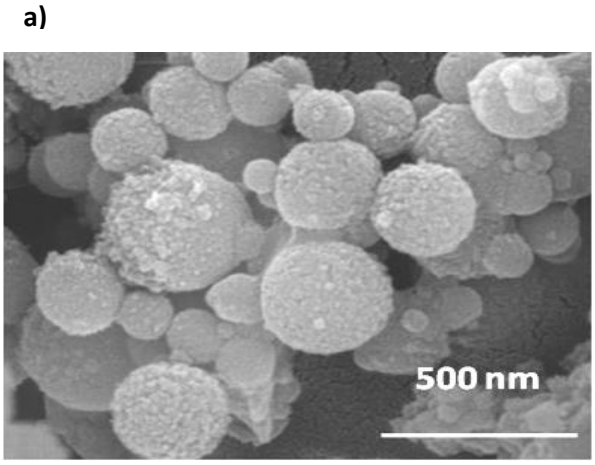
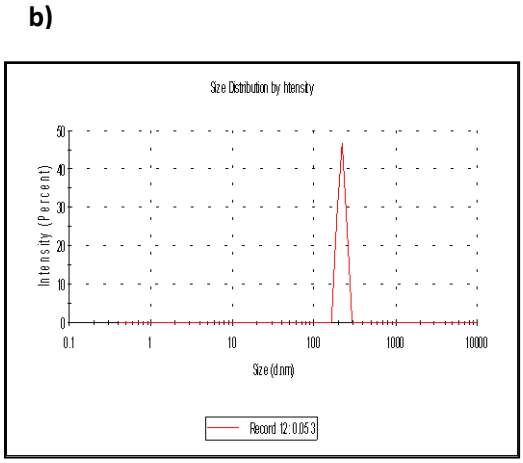


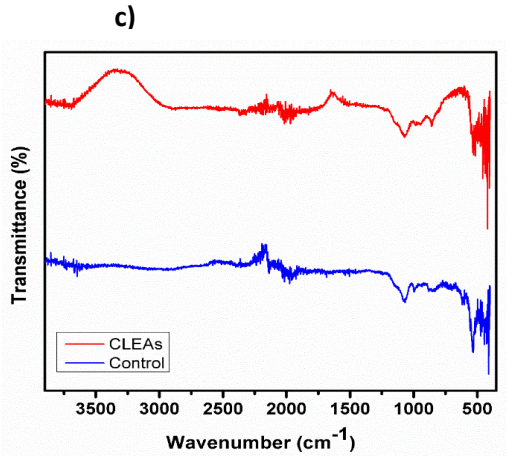
Figure 5 Optimization of Xy-CLEAs preparation. **(a)** Protein concentration **(b)** Precipitant type **(c)** Protein: precipitant ratio **(d)** Precipitation time **(e)** Cross-linker concentration **(f)** Reaction time. All values are mean of three replicates ± SE.

Characterization of Xy-CLEAs

SEM imaging showed spherical structure of Xy-CLEAs with rough and porous surface (Fig.7a) and a diameter of 200-250 nm. DLS spectra of Xy-CLEAs indicated the average particle size of 202 nm (Fig.7b). Particle size is an important property as it has direct correlation with the filterability and mass transfer limitations of CLEAs [38]. FTIR analysis revealed differences between the absorption spectra of free xylanase and Xy-CLEAs (Fig.7c). The change in the position of the peaks confirms the cross-linking which resulted in the conformational alterations of the amide band. Such shifting in the peaks of N-H bond has been reported in earlier studies and it occurs due to alterations in the hydrogen bonds and sulphur bridges within the protein structure [39].







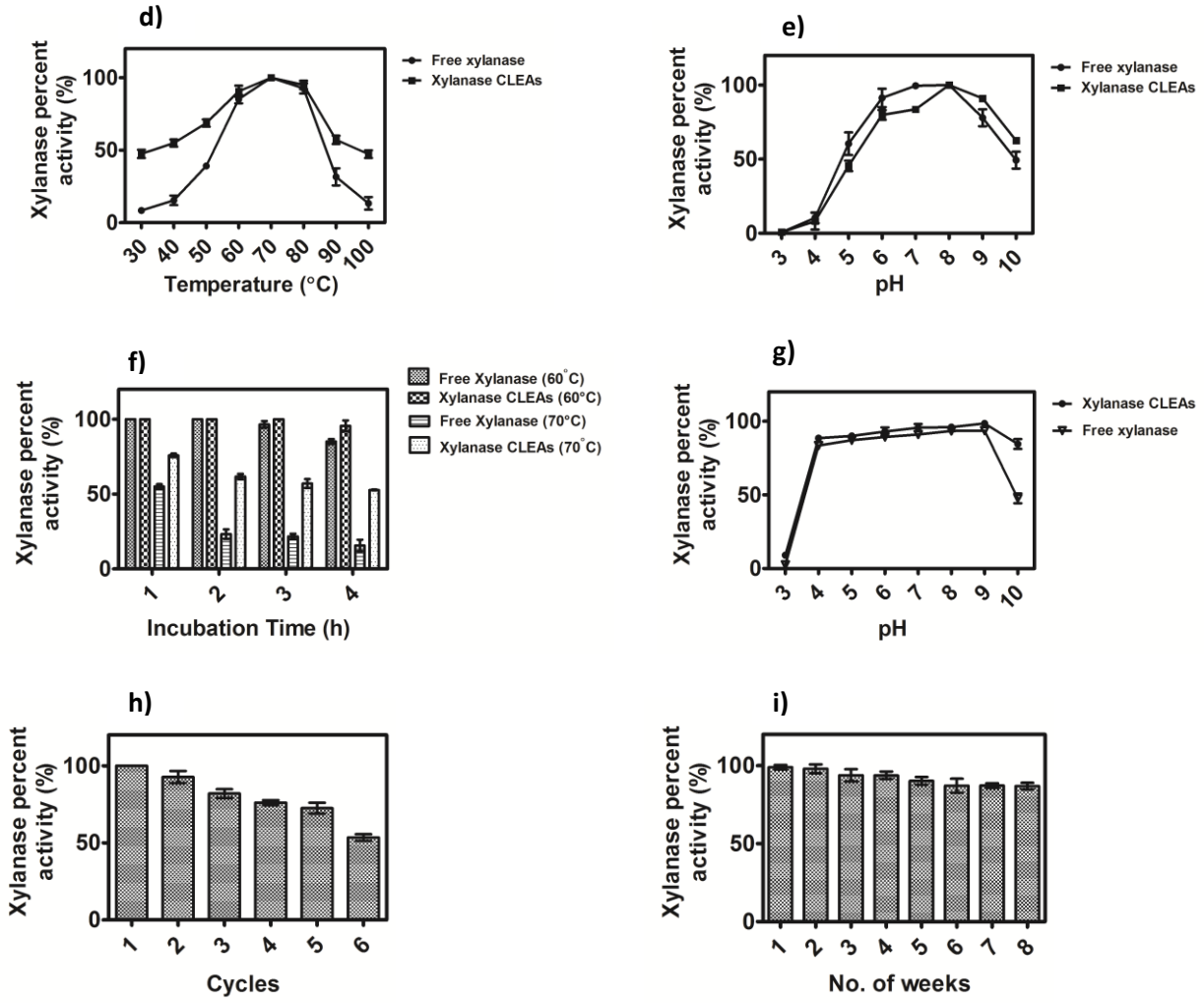


Figure 7 a) SEM image of Xy-CLEAs at magnification 100, 000 x **b)** DLS analysis of Xy-CLEAs **c)** FTIR spectra of free xylanase (control) and Xy-CLEAs **d)** Effect of varying temperature (30-100˚C) on free and immobilized xylanase from *Geobacillus thermodenitrificans* X1 **e)** Effect of varying on free and immobilized xylanase from *Geobacillus thermodenitrificans* X1. **f)** Thermostability of free and immobilized xylanase at 60˚C and 70˚C **g)** Stability of free and immobilized xylanase at different pH (3-10). **h)** Reusability of Xy-CLEAs **i)** Storage stability of Xy-CEAs. All values are mean of three replicates ± SE.

The optimum temperature for both, free and immobilized xylanase was 70˚C (Fig.7c). At higher temperatures (90˚C and 100˚C), xylanase CLEAs showed higher activity (57.2% and 47.3% respectively) in comparison to free xylanase (31.6% and 13.4% respectively) on the same temperatures. Strong covalent bonds of enzyme aggregates within CLEAs protect enzyme from denaturation by heat [40]. The optimum pH for free xylanase was observed at pH 7 and 8, whereas Xy-CLEAs showed maximum xylanase activity (100%) at pH 8 (Fig.7d). The pH shift after immobilization is due to electrostatic modifications in the enzyme occurring due to the

formation of new interactions between the cross-linker (GA) and reactive amino groups on the surface of enzyme aggregates [41].

In the present study, both CLEAs and free xylanase were equally thermostable after 2 h incubation at 60°C (Fig.5e). However, CLEAs showed comparatively higher stability after 3 and 4 h incubation at the same temperature. At 70°C, Xy-CLEAs showed tremendous thermostability and retained 53% after 4 h incubation in comparison to free xylanase that retained only 15% of activity. CLEAs were found to be stable at elevated temperature due to the strong covalent cross-linking within the structure. The kinetic study revealed the deactivation constant (K_d) of free and immobilized xylanase was 0.04 and 0.013 (respectively) at 60°C and 0.465 and 0.161, respectively at 70°C. The $t_{1/2}$ free xylanase was 17 h and 1.5 h at 60°C and 70°C, respectively whereas, immobilized xylanase showed $t_{1/2}$ of 53 h at 60°C and 4 h at 70°C. The immobilized xylanase showed higher stability under the varied pH conditions (3-10) in comparison to the free xylanase (Fig.5f). However, specifically, the percentage difference in the relative activity of the free and immobilized xylanase was higher in the alkaline range (9-10).

Xy-CLEAs retained 53.5% of activity after six repetitive cycles (Fig.5g). With an increasing number of cycles, the relative activity of Xy-CLEAs kept on decreasing. This loss in activity of CLEAs after each cycle was attributed to mechanical shearing and conformational variations of CLEAs due to consecutive centrifugation steps [31]. In addition to reusability, Xy-CLEAs also showed appreciable storage stability (Fig.5h); retaining 86% activity after being stored at 4°C for 8 weeks. The activity of CLEAs is retained even after long term storage, because of limited conformational alterations due to cross-linked matrix.

Objective 4: Application of xylanase for biobleaching of pulp in paper industry

Our study was oriented towards analyzing the potential of xylanase from *Geobacillus thermodenitrificans* X1 in biobleaching of paper. Application of xylanase aids in improving the paper quality and reducing the usage of chlorine for bleaching. With, cost-effective point of view, crude xylanase was used for biobleaching. Xylanase production was carried out in Jaypee University of Information Technology and its application for biobleaching was performed in R&D lab of Kauntum Papers Ltd. situated in Districtt. Hoshiarpur, Punjab. Pulp used

Materials and methods

Pretreatment of agropulp with crude xylanase

The agropulp was distributed as control and test sample. The agropulp considered as test sample (T1; T2- 100 g each) was treated with crude xylanase at a dosage of 50 U/mL for one gram of pulp. In contrast, the control sample (100 g) was not given any xylanase pretreatment. Both the samples were incubated at 70°C for 90 min (Table 3). After incubation, pulp from test and the control sample was examined for pH, kappa no. and brightness, followed by washing with fresh water.

Chlorination and Extraction Stage

T1 and the control sample were treated with 4% chlorine while T2 sample was chlorinated at a dosage of 3.2%. Chlorination of all the samples was carried at ambient temperature for 45 min (Table 4), followed by washing with fresh water. Thereafter, pulp was screened for brightness, initial and final pH after washing. A fraction of pulp from all the samples was pressed into sheets using automatic sheet making machine and dried by sheet dryer. In extraction stage, the chlorinated pulp was treated with 2% caustic soda and hydrogen peroxide (1.32%) at a consistency of 10% (Table 5). Pulp was incubated at 70°C for duration of 120 min, followed by washing with fresh water. Washed pulp was then analyzed to study kappa no. and brightness and also used to make sheets.

Hypo Stage and evaluation of properties of paper

Pulp was treated with sodium hypochlorite solution (available chlorine 2.5%) and 0.1% sulphamic acid. Consistency was maintained at 10.5% and incubation was carried out at 45°C for a period of 120 min (Table 6). Initial and final pH of the pulp was determined along with brightness of the pulp. Paper sheets were prepared and examined for quality and strength properties. Different properties such as freeness, viscosity, basis weight, bulk weight, breaking length, tear factor, and burst factor were evaluated for paper sheets prepared from all the samples (Table 7).

Results and Discussion

Pretreatment of Agropulp with Xylanase

After pretreatment, there was no difference observed in the kappa no. and brightness of control and test samples. This can be justified on the basis of role and action of xylanase on the pulp. Xylanase does not completely remove lignin from the pulp; rather it increases the porosity of pulp by partially hydrolyzing the residual and reprecipitated lignin molecules on the surface of pulp [42]. That is why, the impact of xylanase pretreatment is observed in the subsequent bleaching stages.

Table 3 Pretreatment of agropulp with crude xylanase

DECKER STAGE (XYLANASE PRETREATMENT)				
S.No.	**Particulars**	**Unit**	**Control sample**	**Test sample**
1.	pH	-	8.6	8.6
2.	Kappa no.	-	13.4 ± 0.13	13.4 ± 0.13
3.	Brightness	ISO	38.7 ± 0.34	38.7 ± 0.34
4.	Temperature	°C	70.0	70.0
5.	Enzyme	U/mL/g of pulp	-	50.0
6.	Retention time	Minutes	90	90

Chlorination Stage

After chlorination, T1 sample showed maximum brightness (46.4 ± 0.6 ISO), followed by T2 sample (44.6 ± 0.65 ISO) and control sample (42.8 ± 0.62 ISO). Despite reduction of chlorine dosage by 20% in case of T2 sample, the brightness was greater than that observed in the control sample. These observations create a scope for conducting environment friendly and cost

effective bleaching. Reduction in chlorine consumption due to xylanase pretreatment has been reported in recent studies also. Pretreatment of bagasse pulp by xylanase from *Thermomyces lanuginosus* SSBP resulted in 18% chlorine reduction [43]. Savings of chlorine consumption by 12.5% was observed during pretreatment of wheat straw pulp by xylanase from alkalophilic *Cellulosimicrobium cellulans* CKMX1 [41]. The sheets generated from the chlorinated pulp appeared pale-yellowish in color due to residual lignin.

Table 4 First stage of bleaching, using chlorine as a chemical bleaching agent

CHLORINATION STAGE					
S.No.	**Particulars**	**Unit**	**Control sample**	**Test sample (T1)**	**Test sample (T2)**
1.	Chlorine dosing	%	4.0	4.0	3.2
2.	Consistency	-	3.0	3.0	3.0
3.	Initial pH	-	2.6	2.6	2.8
4.	Temperature	°C	30	30	30
5.	Retention time	Minutes	45	45	45
6.	pH after washing		7.0	7.2	7.3
7.	Brightness	ISO	42.8 ± 0.62	46.4 ± 0.6	44.6 ± 0.65

Extraction Stage

After treatment with caustic soda and hydrogen peroxide, test samples T1 (78.0 ± 0.72 ISO) and T2 (77.0 ± 0.63 ISO) showed greater brightness in comparison to the control (75.2 ± 0.68 ISO). Similar to the present study, the decreased kappa no. and increased brightness in the xylanase treated pulp after alkali extraction has been reported in earlier studies [44,45]. The sheets formed at this stage were brighter and off white in color.

Table 5 Hydrogen-peroxide reinforced extraction of lignin in alkaline environment

EXTRACTION STAGE					
S.No.	**Particulars**	**Unit**	**Control sample**	**Test sample (T1)**	**Test sample (T2)**
1.	Caustic dosing	%	4	4	3.2
2.	H_2O_2 dosing	Kg/ton	12	12	12
3.	Consistency	%	10.1	10.1	10.1
4.	Initial pH	-	11.6	11.3	11.1
5.	Temperature	°C	70	70	70
6.	Retention time	Minutes	120	120	120

7.	Final pH	-	10.5	11.2	11.0
8.	Kappa no.	-	1.5 ± 0.15	1.14 ± 0.17	1.3 ± 0.13
9.	Brightness	ISO	75.2 ± 0.68	78.0 ± 0.72	77.0 ± 0.63

Hypo Stage

Xylanase pretreated samples, T1 and T2 showed 85 ± 0.74 ISO and 84 ± 0.8 ISO brightness, respectively. Whereas, control sample rendered lower brightness; showing 82.8 ± 0.7 ISO. So, final brightness achieved was >80 ISO with an increase of 2.2 points in T1 sample and 1.2 points in T2 sample in comparison to the control. Moreover, achieving >80 ISO brightness after reducing the amount of chlorine by 20%, is appreciable from commercial point of view. Many other literature reports are in accordance with the observations of the present study [46,47]. Eventually, the sheets generated were evaluated for physical and strength properties.

Table 6 Final stage of bleaching, using sodium hypochlorite as bleaching agent and sulphamic acid as an enhancer of this bleaching reaction

HYPO STAGE					
S.No.	**Particulars**	**Unit**	**Control sample**	**Test sample (T1)**	**Test sample (T2)**
1.	Hypochlorite dosing	%	2.5	2.5	2.5
2.	Sulphamic acid	%	0.1	0.1	0.1
3.	Consistency	%	10.5	10.5	10.5
4.	Initial pH	-	9.2	9.2	9.3
5.	Temperature	°C	45	45	45
6.	Retention time	Minutes	120	120	120
7.	Final pH	-	6.65	6.75	6.75
8.	Brightness	ISO	82.8 ± 0.7	85 ± 0.74	84 ± 0.8

Evaluation of properties of paper

The properties like basis weight, bulk weight, burst factor and viscosity in case of test samples were comparable to that of control whereas freeness breaking length and tear factor were increased (Table 7). On the other hand, on reducing the dosage of chlorine by 20% for the xylanase pretreated pulp (T2) there was appreciable increase observed for the strength properties like breaking length and tear factor. Viscosity and bulk weight were comparable, while burst factor decreased. Freeness was increased by 1 point and basis wt. was decreased by 1 point.

Table 7 Evaluation of properties of the paper generated from agropulp for assessment of paper quality and efficiency of bleaching method

PHYSICAL AND STRENGTH PROPERTIES					
S.No.	**Property**	**Unit**	**Control sample**	**Test sample (T1)**	**Test sample (T2)**
1.	Freeness	°SR	21 ± 0.32	22 ± 0.25	22 ± 0.2
2.	Basis weight	gm/m^2	61.0 ± 0.4	61.2 ± 0.4	60 ± 0.6
3.	Bulk weight	cc/gm	1.67 ± 0.1	1.66 ± 0.13	1.64 ± 0.1
4.	Breaking length	meter	3865 ± 7.2	4100 ± 4.7	4065 ± 4.8
5.	Tear factor	-	45.3 ± 0.74	55.2 ± 0.82	53.8 ± 0.8
6.	Burst factor	-	23.2 ± 0.4	23.0 ± 0.47	22.4 ± 0.36
7.	Viscosity	cps	7.3 ± 0.25	7.3 ± 0.32	7.28 ± 0.27

Conclusion

The current study elucidates the potential of *Geobacillus thermodenitrificans* X1 for effective xylanase production, using wheat straw as a carbon source. The exploitation of lignocellulosic residues for enzyme production makes the process economical and environment-friendly. The thermostable, alkali-stable and cellulase free properties of the xylanase warrants its application in paper pulp industry. The xylanase was highly active on xylan and effectively liberated XOs from hydrolysis of xylan. The new isolate of *G. thermodenitrificans* emerged out to be potent producer of thermo-alkali-stable xylanase under cost-effective conditions. For enhancing commercial viability crude thermo-alkali-stable xylanase from *Geobacillus thermodenitrficans* X1 was successfully immobilized to form CLEAs. Significant properties such as pH and thermal stability were tremendously improved and the Xy-CLEAs also rendered efficient reusability and storage stability. The improved characteristic features of Xy-CLEAs in the present study highlights the promising potential of Xy-CLEAs to be used economically on the industrial scale. However, loss of activity is the only drawback, which needs to be overcome. Moreover, being thermo-alkali-stable, GTX1 xylanase was able to withstand high temperature and pH conditions of pulp biobleaching process in paper industry. GTX1 xylanase had no deteriorating effect on the cellulose structure, which was evident from its negligible effect on the viscosity of paper. Overall, the quality of the paper was improved after xylanase pretreatment. Thus GTX1 xylanase comes out to be commercially viable biobleaching agent by reducing the use of chlorine dosage for bleaching and thus curbing the environmental impact.

Future Prospects

The titer of xylanase produced from *Geobacillus thermodenitrificans* X1 could be further increased by opting molecular techniques. Heterologous or homologous overexpression of xylanase gene could probably trigger the enhanced production; as observed in the literature reports. The study could be followed by purification and characterization of recombinant GTX1 xylanase and also the comparative study between the xylanase from wild and the recombinant one.

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List of publications

Journal Publications

1. **R. Verma**, A. Bhalla and S. Kumar. "Valorization of Lignocellulosic Residues for Cost-Effective Production of Thermo-Alkali-Stable Xylanase by *Geobacillus thermodenitrificans* X1 of Indian Himalayan Hot Spring", *Waste Biomass Valorization*, pp.1-11, July 2018. **IF: 1.87 [Scopus]**

2. **R. Verma**, A. Kumar and S. Kumar. "Synthesis and characterization of cross-linked enzyme aggregates (CLEAs) of thermostable xylanase from *Geobacillus thermodenitrificans* X1", *Process Biochem.*, vol. 80, pp. 72-79, Jan. 2019. **IF: 2.6 [Scopus]**

3. **R. Verma**, S. Chechi and S. Kumar. "Biobleaching of wheat straw-bagasse pulp using thermo-alkali-stable xylanase from *Geobacillus thermodenitrificans* X1", *Waste Biomass Valorization*. (Under review) **IF: 1.87 [Scopus]**

4. **R. Verma**, A. K. Tripathi, A. Yadav and S. Kumar. "Comparative analysis of various pretreatment methods for sugar recovery from waste foliage of *Pinus roxburghii* and *Pinus wallichiana*". (Under preparation)

Conference paper

R. Verma and A. Kumar, "Biogas Plants for Community Service", in *National Biogas convention for Sustainable Energy Access in Rural Areas: Proc. of 2nd National Conf. on Current and Emerging Trends in Indian Biogas and Bio-Fertilizer Development, CETIBBD-2015, September 15-17, 2015, Indian Institute of Technology Delhi, Hauz Khas, New Delhi-110016*, V. K. Vijay, R. Chandra, A. Trivedi, B. Jha, V. Vijay, Eds. IIT Delhi, 2015. pp. 113-122.

Book Chapter

R. Verma, A. K. Tripathi and S. Kumar, "Conversion of Lignocellulosic Feedstocks into Biogas" in *Extremophilic Microbial Processing of Lignocellulosic Feedstocks to Biofuels, Value-Added Products, and Usable Power*, 1st ed. R. Sani and R. N. Krishnaraj, Ed. Switzerland:Springer Nature, 2018, pp. 111-143.

Correspondence article

R. Verma, A. Kumar and S. Kumar, "CO_2 levels and Coral Reefs", *Curr. Sci.*, vol. 111 (8), pp. 1288, Oct. 2016

NCBI GenBank submissions

Verma R and Kumar S (2015): KT899095

Verma R and Kumar S (2018): MG874777, AYF56152

Conference Abstracts

1. W. Sharma, **R. Verma** and S. Kumar. "Process optimization for xylanase production using thermophilic bacteria isolated from biogas slurry". 2nd Himachal Pradesh science Congress (HPSC) 2017-18 organized by Himachal Pradesh Council for Science, Technology & Environment (HIMCOSTE), held at Hotel Peterhoff, Shimla, Nov. 20-21st , 2017.

2. P. Sethi, **R. Verma** and S. Kumar. "Bioprospecting for xylanse producing bacteria with potent application in lignocellulosic conversion". 2nd International Conference: Innovative Research in Engineering, Science and Technology (IREST-2017) held at Eternal University during April 7-8, 2017.

3. **R. Verma**, A. Bhalla and S. Kumar. "Isolation of thermophilic xylanase from hot spring soil sample". Second International Conference on 'Recent Advances in Bio-energy Research', held at National Institute of Bioenergy, Kapurthala, Jalandhar, Feb. 25-27, 2016.

4. **R. Verma**, A. Bhalla and S. Kumar. "Extremophilic xylanases for efficient conversion of lignocellulosic biomass". Second International Conference on 'Recent Advances in Bio-energy Research', held at National Institute of Bioenergy, Kapurthala, Jalandhar, Feb. 25-27, 2016.

5. **R. Verma**, Anil Kumar, Abhilash Kumar Tripathi, Ashish Kumar and Sudhir Kumar. "Biogas for community services". National biogas convention 2015 on Current and emerging trends in Indian biogas and bio-fertilizer development, organized by biogas development and training center, center for rural development and technology, IIT Delhi, Sept. 15-16, 2015.

Trainings and Workshops

1. Participated in one day workshop on "Patent Drafting" held on 13th October 2017 at Jaypee Uiversity of Information Technology, Wakanghat, Solan, Himachal Pradesh.

2. Participated in one day workshop on "Innovation and Intellectual Property Rights" held on 9th December 2016 at Jaypee Uiversity of Information Technology, Wakanghat, Solan, Himachal Pradesh.

3. Participated in one week training program on Hands on Analytical and Molecular Tehniques 'Biomass based Biorefineries: An Emerging Incentive for Advanced Biofuels and Value-added Products' during February 23-27, 2015.